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$CD4^+CD25^+Foxp3^+$ Regulatory T Cells Promote Th17 Cells In Vitro and Enhance Host Resistance in Mouse *Candida albicans* Th17 Cell Infection Model

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SUMMARY

Th17 cells and $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells are thought to promote and suppress inflammatory responses, respectively. Here we explore why under Th17 cell polarizing conditions, Treg cells did not suppress, but rather upregulated, the expression of interleukin-17A (IL-17A), IL-17F, and IL-22 from responding $CD4^+$ T cells (Tresp cells). Upregulation of IL-17 cytokines in Tresp cells was dependent on consumption of IL-2 by Treg cells, especially at early time points both in vitro and in vivo. During an oral *Candida albicans* infection in mice, Treg cells induced IL-17 cytokines in Tresp cells, which markedly enhanced fungal clearance and recovery from infection. These findings show how Treg cells can promote acute Th17 cell responses to suppress mucosal fungus infections and reveal that Treg cells have a powerful capability to fight infections besides their role in maintaining tolerance or immune homeostasis.

INTRODUCTION

$CD4^+CD25^+Foxp3^+$ T cells, termed regulatory T (Treg) cells, are thought to be a stable lineage of cells that plays an active suppressive role in the maintenance of immunological self-tolerance and immune homeostasis, but whose role in protective immunity is not fully understood (Sakaguchi et al., 2009). The suppressive functions are exhibited in Foxp3-deficient mice and the human "immune dysregulation enteropathy polyendocrinopathy X-linked" (IPEX) syndrome patients that succumb to fatal inflammatory disorders associated with fewer numbers of Treg cells (Ochs et al., 2007). Interestingly, although IPEX patients manifest apparent autoimmune diseases, they also have a susceptibility to specific infectious diseases, notably *Candida albicans* infections, suggesting selective immunodeficiency (Ochs et al., 2007). The transcriptional repressive effects of the forkhead box P3 (Foxp3) protein render Treg cells incapable of producing certain key cytokines such as interleukin-2 (IL-2) and so they require an exogenous supply of these cytokines for their peripheral maintenance (Pandiyan and Lenardo, 2008). Indeed, Treg cells compete for IL-2 and other survival cytokines, leading to cytokine deprivation apoptosis of effector T cells (Pandiyan et al., 2007). Careful experimental modeling of the cytokine competition mechanism of suppression by Treg cells reveals that suppression depends strongly on the local cytokine milieu and the proximity of Treg cells to effector cells during an immune response (Busse et al., 2010; Tang and Bluestone, 2008). Treg cells may not effectively suppress by cytokine competition when cytokines are abundant such as during an infection. Some studies have predicted that Treg cells could lose their suppressive functions during acute inflammation in microbial infection models (Oldenhove et al., 2009; Tsuji et al., 2009). Plasticity of Treg cells and their potential nonsuppressive immune functions have been the recent focus of speculation (Zhou et al., 2009). Importantly, certain investigations have demonstrated protective functions for Treg cells during viral infections (Lanteri et al., 2009; Lund et al., 2008). Thus, whether Treg cells may have broader roles in immunity than just the previously recognized suppressor functions is a key area for further discovery.

T helper 17 (Th17) cells produce abundant inflammatory cytokines and are key mediators in host defense, inflammatory disorders, and autoimmune conditions (Korn et al., 2009). Mechanisms of interactions between Treg cells and Th17 cells and the paradoxical ability of Treg cells to augment IL-17A induction are not well understood in vivo (Veldhoen et al., 2006; Xu et al., 2007). Therefore, we chose to study Treg cell function in the context of differentiating Th17 cells. One of the most important functions of Th17 cells in host immunity is to protect against fungal infections. Oropharyngeal candidiasis or "thrush," an acquired immune deficiency syndrome-defining illness, is an infection by the commensal fungus *C. albicans* (Conti et al., 2009). It has been well documented in mice and humans that Th17 cells and IL-17 production are critical for oral fungicidal immune responses by recruiting neutrophils to the oral mucosa and inducing salivary antimicrobial factors (Conti et al., 2009; Curtis and Way, 2009; Eyerich et al., 2008). Patients with hyper-IgE syndrome with fingernail candidiasis or chronic mucocutaneous candidiasis have impaired Th17 cell responses (Milner et al., 2008). Interestingly, patients lacking Treg cells, including IPEX patients, those with IPEX-like syndrome (CD25-deficient patients) or autoimmune polyendocrinopathy-candidiasis

ectodermal dystrophy (APECED) patients deficient in the autoimmune regulator (AIRE) protein, also are highly susceptible to *C. albicans* infections (Kekäläinen et al., 2007; Roifman, 2000). The underlying mechanism and possible roles of Treg cell deficiency in this susceptibility are unclear (Coutinho and Carneiro-Sampaio, 2008; Kekäläinen et al., 2007; Ochs et al., 2009). Therefore, we chose to investigate the function of Treg cells in modulating Th17 cell responses in an oral *C. albicans* infection model.

Here we showed that Treg cells can powerfully promote the transition of naive CD4 cells to Th17 cells producing the full suite of characteristic cytokines independently of the effect mediated by transforming growth factor (TGF)-β (Veldhoen et al., 2006; Xu et al., 2007). Treg cells achieved this by consuming IL-2 and thereby preventing it from inhibiting Th17 cell differentiation both in vitro and in vivo. Treg cells did not suppress, but actually promoted, IL-17A-dependent clearance of fungi during acute *C. albicans* infection. However, despite contributing potently to this acute immunoprotective effect, Treg cells exhibited suppressive properties at late times and inhibited chronic Th17 cell-mediated inflammatory bowel disease (IBD). Thus, we provide insights into a facet of Treg cell biology and that, in addition to immune suppression, they promote Th17 cell differentiation and participate in host protective immunity against fungal infections such as by *C. albicans*.

RESULTS

Treg Cells Upregulate Th17 Cell Cytokines from Responder T Cells In Vitro

Previous studies have shown that TGF-β provided by Treg cells is essential for IL-17A induction in naive CD4 cells stimulated with dendritic cells and IL-6 (Veldhoen et al., 2006; Xu et al., 2007). In order to study how Treg cells contributed to the induction of IL-17A in CD4 cells via Th17 cell polarizing conditions (Korn et al., 2009), we stimulated $CD4^+CD44^{lo}CD62L^{hi}CD25^-$ naive (Tresp) cells in the presence of control green fluorescent protein $(GFP)^-CD4^+CD44^{lo}CD25^-$ (>99% purity) (Tcon) or $GFP^+CD4^+CD25^+$ Treg (>99% purity) cells flow cytometrically sorted from *Foxp3*^{gfp} reporter mice. Tcon or Treg cells were derived from CD45.2 B6 mice and Tresp (equivalent to Tcon) cells were derived from CD45.1 congenic mice so that Tresp cells could be selectively identified via CD45.1 staining (Figure S1 available online). Under optimal Th17 cell polarizing conditions, we were surprised to observe that the fraction of IL-17A producers, as assessed by intracellular staining and the mean fluorescence intensity (MFI) of IL-17A in Tresp cells, was still significantly boosted by cocultivation with Treg cells compared to those cultured alone or with Tcon cells on day 3 (d3) (Figure 1A). We confirmed these findings by using flow cytometry-sorted $CD4^+CD25^+$ Treg cells (>99% purity) and $CD4^+CD44^{lo}CD25^-$ Tcon cell preparations in five independent experiments (Figures 1B and 1D, top). Tresp cells cocultured with Treg cells increased the frequency of IL-17A producers also at time points as late as day 7 (Figure 1D, bottom; Figure S2A). CD3-CD28 restimulated Tresp cells also showed increased IL-17A in Treg cell cocultures compared to controls (Figure S2B). Induction of IL-17A was a specific property of $CD25^+Foxp3^+$ Treg cells as shown by the fact that activated $CD25^+$ effector ($CD4^+CD25^+$ Teff) cells did not upregulate IL-17A in naive Tresp cells (Figure S2C). We also found that cytokines such as IL-17F and IL-22 were boosted by the presence of Treg cells, indicating that the full differentiation program of Th17 cells was being promoted both on d4 and d6 (Figures 1C and 1E; Figure S2D). Among Tresp cells, there were very few $Foxp3^+$ and IFN-γ^+ cells irrespective of Treg cell addition, showing that our cultures had bona fide Th17 cells and that Treg cells did not skew them toward induced Treg (iTreg) cell or other lineages (Figures 1C and 1E; Figure S2D). Supernatants derived from these cells also showed increased amounts of IL-17A and IL-21 both at early and late time points (Figure 1F). We also examined CD45.2 Tcon and Treg cells in Th17 cell coculture conditions. Although Tcon cells were similar to Tresp cells, 30% of Treg cells lost Foxp3 expression and 5% of those cells expressed IL-17A on d3 (Figure S3A). Control CD45.2 Treg cells that were cultured alone with IL-2 under Th17 cell polarizing conditions did not lose Foxp3 (Figure S3A, right). When we followed Treg cells in Th17 cell cocultures at different time points, we found that the frequency of $Foxp3^+$ cells that started as >99% dropped to 50% on d3 and rebounded to about 90% on d7 (Figure S3B). Among Treg cells, we observed an initial increase in the frequency of $IL\text{-}17A^+Foxp3^-$ cells (12%), which seemed unlikely to be due to outgrowth of contaminating Th17 cells because these decreased in number as Foxp3 expression was restored over the next few days. Further work will be required to verify that cells actually altered phenotype as these data imply. Taken together, these results reveal that Treg cells play one or more role(s) in promoting the induction of fully differentiated Th17 cells, thereby verifying and extending the previous findings demonstrating the positive effect of Treg cells on Th17 cell differentiation (Veldhoen et al., 2006; Xu et al., 2007).

Treg Cells Consume IL-2 to Induce IL-17A and IL-17F in Tresp Cells

In the study by Veldhoen et al. (2006), the stimulatory effect of TGF-β on IL-17A production was saturated at a concentration of 0.5–1 ng/ml, whereas we used an excess of TGF-β (2 ng/ml). We also found that the increase in IL-17A by TGF-β was saturated at 1 ng/ml in our cultures (Figure S4A). Moreover, supernatant alone from Treg cell cultures and Treg cells across a transwell dish did not induce IL-17A in Tresp cells (Figure S4B). Treg cells enhanced IL-17A in cells that were cultured in a Th17 cell milieu both in normal media and in serum-free media, therefore ruling out any effect of TGF-β in the serum (Figures S4B and S4C). These data indicated that under saturating concentrations of TGF-β, Treg cells upregulated IL-17A in Tresp cells independently of TGF-β from Treg cells. Consequently, we speculated that there must be another mechanism for IL-17A enhancement by Treg cells.

IL-2 has been shown to suppress IL-17A production through a STAT-5-dependent mechanism (Laurence et al., 2007). Meanwhile, it has been previously demonstrated that Treg cells are potent consumers of IL-2 (Maloy and Powrie, 2005) and IL-2 deprivation is a fundamental aspect of their suppressive capability (Maloy and Powrie, 2005; Pandiyan and Lenardo, 2008). Therefore, we speculated that Treg cells could be increasing IL-17A production by removing the inhibitory effect of IL-2. We therefore added exogenous IL-2 to Tresp cells under Th17

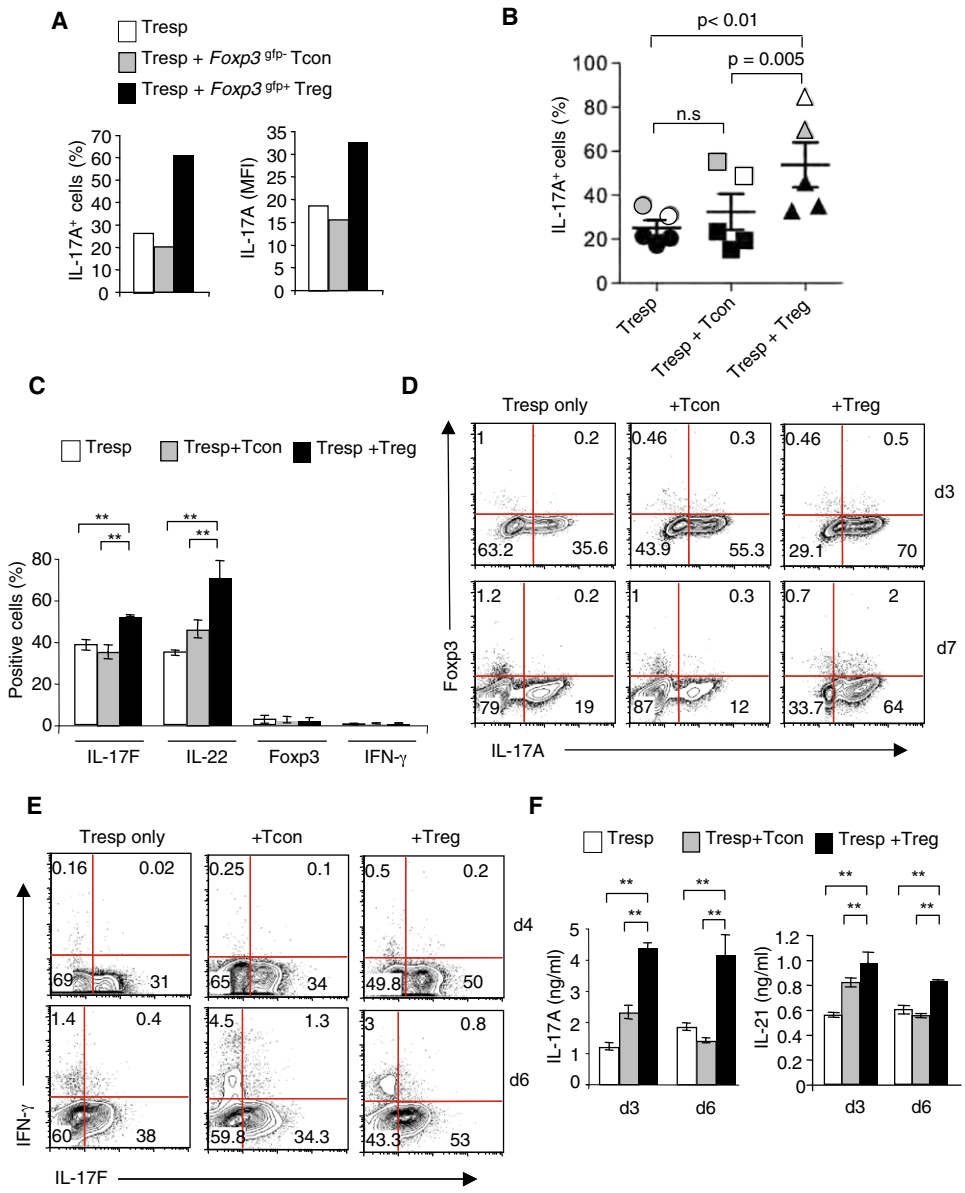

Figure 1. Upregulation of Th17 Cell-Associated Cytokines in Tresp Cells by Treg Cells

(A) CD45.1 Tresp cells were cultured alone or cocultured with Foxp3$^-$GFP$^-$CD45.2 Tcon or Foxp3$^-$GFP$^-$ Treg cells under Th17 cell polarizing conditions for 3 days. Intracellular cytokine staining was performed in PMA-ionomycin restimulated cells. Data from flow cytometric analyses (gated on CD45.1$^+$ cells) shows the percentage of IL-17A$^+$ cells (left) and mean fluorescence intensities (MFI) of IL-17A (right).

(B) The frequency of IL-17A$^+$ cells at d3 or d4 from five independent experiments is depicted. Data points ± SEM are plotted. Grey and white data points each indicate separate experiments. Black points are from three independent experiments.

(C) Cells stimulated as in (B) were stained for IL-17F, IL-22, Foxp3, or IFN-γ and the data showing the percentage of respective cytokine-positive cells are plotted (right).

(D and E) Flow cytometric dot plots (gated on CD45.1$^+$ cells) of IL-17A and Foxp3 staining (D) and IL-17F and IFN-γ (E) are shown in x and y axes, respectively.

(F) ELISA quantification of IL-17A (left) and IL-21 (right) in the supernatants of indicated cultures stimulated under Th17 cell polarizing conditions.

**p < 0.05.

cell-inducing culture conditions both in the presence and absence of Treg cells. We found that IL-17A production with or without Treg cells was drastically reduced (Figure 2A; Figure S4D). In parallel experiments, we observed that *Il2rb*$^{-/-}$ mice had higher amounts of IL-17A in their serum compared to wild-type (WT) mice, suggesting that a suppressive effect of IL-2 on IL-17A production can also occur in vivo (Figure S4E). These data support the general principle that IL-2 suppresses IL-17A production under physiological conditions (Laurence et al., 2007). Because we hypothesized that in the presence of excess TGF-β, IL-2 consumption by Treg cells was responsible for the enhancement of Th17 cell differentiation, we quantified

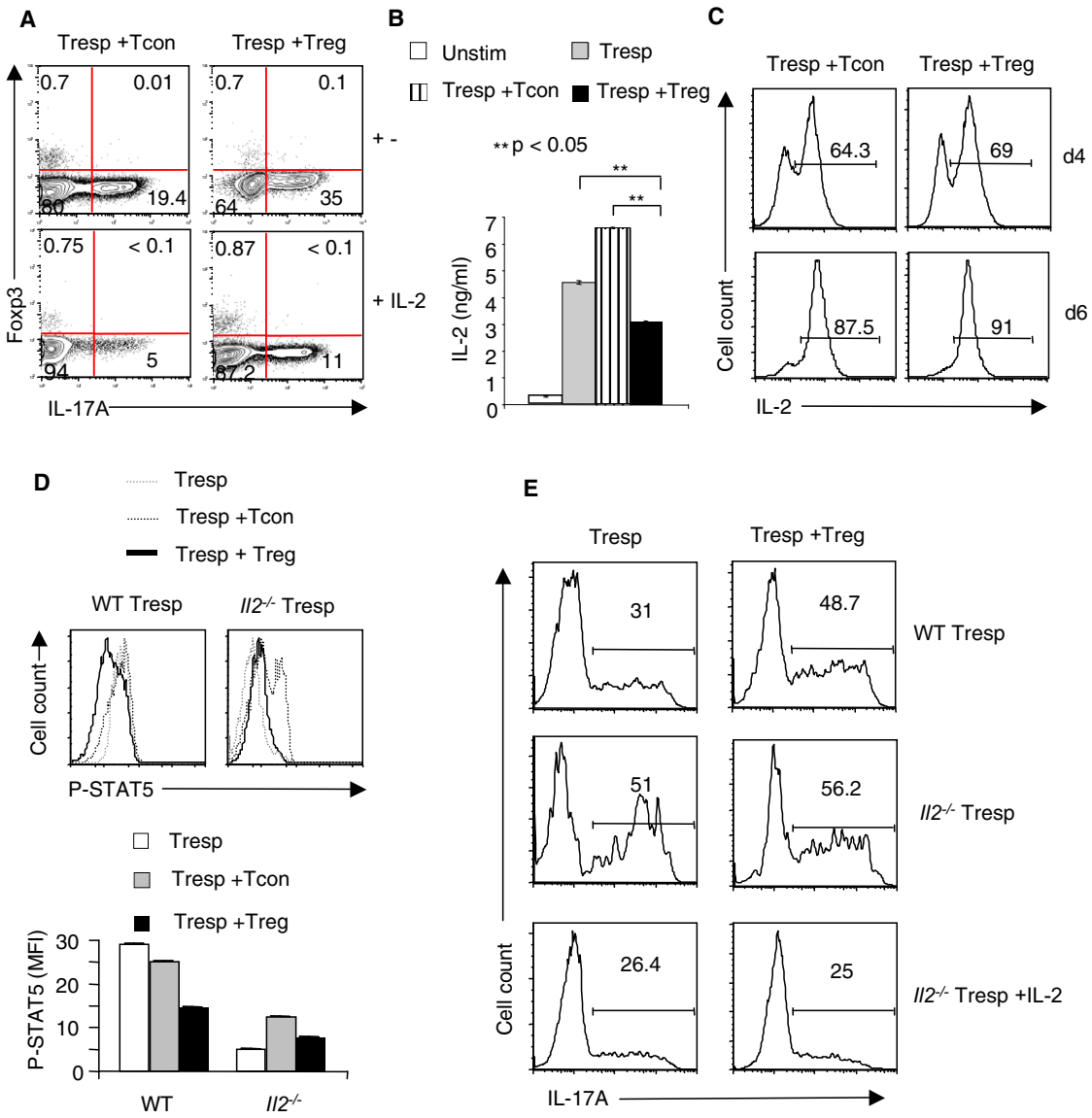

Figure 2. Induction of IL-17 in Tresp Cells Is Dependent on IL-2 Consumption by Treg Cells on Day 3

(A) Tresp cells were stimulated under Th17 cell skewing conditions with Tcon or Treg cells as in Figure 1B. Intracellular staining of IL-17A and Foxp3 in Tresp cells with Tcon or with Treg cells that were stimulated for 3 days with or without IL-2 added at the beginning of stimulation.

(B) Loss of IL-2 accumulation in Treg-Tresp Th17 cell cocultures. ELISA quantification of IL-2 in unstimulated culture (open bar), in Tresp alone (light gray), Tcon (striped), or in Treg (black) cell cocultures stimulated for 4 days. Results represent the mean ± SD.

(C) The percentage of IL-2$^+$ Tresp cells in cocultures is shown. The results are representative of at least three independent experiments.

(D) Flow cytometric histograms of intracellular P-STAT5 staining of WT or *Il2*$^{-/-}$ Tresp cells (top) showing the MFI of the P-STAT5 staining (bottom) in the indicated cultures.

(E) Intracellular IL-17A staining of WT and *Il2*$^{-/-}$ Tresp cells with or without Treg cells or 100 U/ml of IL-2. At least three independent experiments showed similar results.

IL-2 in the supernatants by ELISA. We found that IL-2 was reduced in Treg cell cocultures compared to Tresp cells alone or Tcon cell controls, consistent with our hypothesis (Figure 2B). Because we stimulated the cells with α-CD3 and α-CD28 in the absence of antigen-presenting cells, IL-2 synthesis by Tresp cells was unaffected by Treg cells (Figure 2C). These data indicate that the reduced amounts of IL-2 were most probably attributable to consumption by Treg cells rather than suppressed IL-2 production, consistent with previous reports for such culture systems (de la Rosa et al., 2004; Pandiyan et al., 2007).

We also looked for other molecular concomitants in Tresp cells reflecting IL-2 consumption in Treg cell cocultures. Decreased IL-2 receptor (CD25) expression and decreased STAT-5 signaling are clear manifestations of T cells experiencing reduced IL-2 signals (Laurence et al., 2007). In our cocultures, we found that the presence of Treg cells caused decreased CD25

expression and markedly reduced phosphorylation of STAT-5 (P-STAT5), indicating that IL-2 was sufficiently reduced to cause signaling changes in Tresp cells (Figure 2D; Figure S4F). To further examine the effects of IL-2 in our Th17 cell cultures, we stimulated *Il2*$^{-/-}$ Tresp cells with WT Tcon or Treg cells. We found that *Il2*$^{-/-}$ Tresp cells alone displayed very low P-STAT-5 amounts and was not further reduced by addition of Treg cells (Figure 2D). On the other hand, Tresp cells cultured with WT Tcon cells induced P-STAT-5 modestly, presumably because of the IL-2 produced by the latter (Figure 2D). More strikingly, we found that *Il2*$^{-/-}$ Tresp cells generated a high fraction of Th17 cells that was hardly affected by Treg cells (Figure 2E). Similarly, Treg cell-mediated upregulation of IL-17F was also less prominent when cultured with *Il2*$^{-/-}$ Tresp cells (Figure S4G). Furthermore, the addition of exogenous IL-2 to the cocultures containing *Il2*$^{-/-}$ Tresp cells reduced the frequency of IL-17A-producing cells and abrogated any inducing effect of Treg cells (Figure 2E). *Il2*$^{-/-}$ cells did not have an enhanced ability to produce cytokines as indicated by the fact that the fraction of cells producing IFN-γ was the same as WT cells (Figure S4G, *y* axis). Hence, these data are consistent with the possibility that IL-2 consumption by Treg cells plays an important role in the upregulation of IL-17 cytokines in Tresp cells on day 3. However, on day 6 after stimulation, additional effects of Treg cells on IL-17 production were observed (compare Figure S4H to Figure 2E). At that later time point, Treg cells increased IL-17A in WT and *Il2*$^{-/-}$ cells, both in the presence and absence of exogenous TGF-β (Figures S4H and S4I, top and middle). This could probably be due to TGF-β secretion by Treg cells increasing IL-17A release from WT and *Il2*$^{-/-}$ cells when exogenous TGF-β is limiting (Veldhoen et al., 2006; Xu et al., 2007). We tested this conjecture by adding α-TGF-β and found that blocking TGF-β abrogated the Treg cell-mediated increase in the frequency of IL-17A-producing Tresp cells (Figures S4H and S4I, middle and bottom). These results show that the effect of Treg cells on Th17 cell differentiation is independent of IL-2 consumption at later time points.

Treg Cell-Dependent IL-2 Consumption Does Not Regulate Survival in Th17 Tresp Cells

To examine whether IL-2 consumption by Treg cells also regulates the survival and proliferation of Tresp cells under Th17 cell polarizing conditions, we assessed the proliferation of the Tresp cells stimulated for 4 days. As a positive control, we also stimulated Tresp cells under neutral conditions (Th0). Consistent with our previous findings (Pandiyan et al., 2007), Th0 cells showed retarded proliferation and prominent apoptosis after 4 days of coculture with Treg cells, whereas Tresp cells under Th17 cell polarizing conditions proliferated extensively and did not undergo cell death (Figures 3A–3C). Thus, Treg cells clearly do not suppress Th17 cells by inducing apoptosis at early time points as they do with other classes of CD4^{+} T lymphocytes. Intracellular staining of IL-2 revealed that the frequency of IL-2 producers and MFI of IL-2 were greatly increased in Th17 cells compared to Th0 cells (Figure 3D). Thus, Treg cells were ostensibly incapable of consuming sufficient IL-2 to cause apoptosis in Th17 cells (Figure 2B). On the other hand, lower IL-2 production in Th0 cells appears to render them susceptible to IL-2 deprivation apoptosis (Pandiyan et al., 2007). Furthermore, whereas exogenous addition of IL-2 suppressed the differentiation of Th17 cells, it increased the frequency of IFN-γ producers in Th0 cells (Figure 3E). This shows that whereas IL-2 provides trophic sustenance and stimulates proliferation of both Th0 and Th17 cells, it apparently has opposite effects on their differentiation. This may explain why IL-2 consumption by Treg cells has differential effects on these two lineages. These findings also verify that Treg cell-dependent IL-17A induction in Tresp cells is not due to altered survival or proliferation of differentiating Th17 cells. Taken together, our data reveal that IL-2 consumption by Treg cells is apparently insufficient to impact Th17 cell survival but removes an inhibitory effect on Th17 cell differentiation.

Treg Cells Enhance Th17 Cell Differentiation by IL-2 Consumption In Vivo

We hypothesized that Treg cells may promote Th17 cell differentiation in vivo in a manner similar to what we observed in vitro and sought to examine the effects of IL-2 and Treg cells in the B10A 5CC7 T cell receptor (TCR) transgenic (tg) model. To examine whether Treg cells regulate Th17 cell differentiation by IL-2 consumption in vivo, we transferred 6 × 10^{5} carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4^{+}CD25^{-} cells (Tresp cells) from congenic CD45.1 WT or *Il2*$^{-/-}$ 5CC7 *Rag2*$^{-/-}$ TCR tg mice in to B10A *Rag2*$^{-/-}$ mice and determined their IL-17A production in response to immunization with 20 μg of pigeon cytochrome C (PCC) peptide emulsified in 200 μl of complete freund's adjuvant (CFA) (200 μg of *Mycobacterium tuberculosis*). Some mice received phosphate-buffered saline (PBS) or 1 × 10^{5} fresh Treg cells from donor B10A 5CC7 TCR tg mice. Four days after immunization, we found that WT 5CC7 TCR tg cells that were cotransferred with Treg cells showed increased IL-17A production in the spleen and lymph nodes (Figure 4A). The frequency of IL-17A producers was increased in *Il2*$^{-/-}$ cells significantly above WT cells and was not affected by the addition of Treg cells (Figures 4A and 4C, *y* axis). IL-2 production was absent in *Il2*$^{-/-}$ cells, but the frequency of IL-2 producers in WT Tresp cells was not decreased by the presence of Treg cells, showing that Treg cells did not suppress IL-2 production in vivo (Figure 4B). We also found that the frequency of IFN-γ producers was unchanged in the presence or absence of Treg cells, showing that they did not promote Th17 cell differentiation by suppressing IFN-γ in vivo (Figure 4C, *x* axis). Because Treg cells did not suppress IL-2 production in WT cells and did not affect Th17 cell differentiation in *Il2*$^{-/-}$ cells, consistent with our in vitro data, we surmised that Treg cells probably enhanced IL-17 production by consuming IL-2 in vivo.

Treg Cells Enhance Th17 Cell Response and the Clearance of *C. albicans* Infection In Vivo

Next we sought to examine the effects of IL-2 and Treg cells in a *C. albicans* infection model. Oropharyngeal candidiasis is an infection by the commensal fungus *C. albicans* and resistance to oral *C. albicans* infection requires a protective Th17 cell response in mice (Conti et al., 2009). Furthermore, IFN-γ-deficient mice clear the infection efficiently, showing that Th1 cells play little or no role in fungal clearance (Farah et al., 2006). *C. albicans* infection in mice is characterized by fungal lesions and inflammation in the tongue, decreased food intake, weight

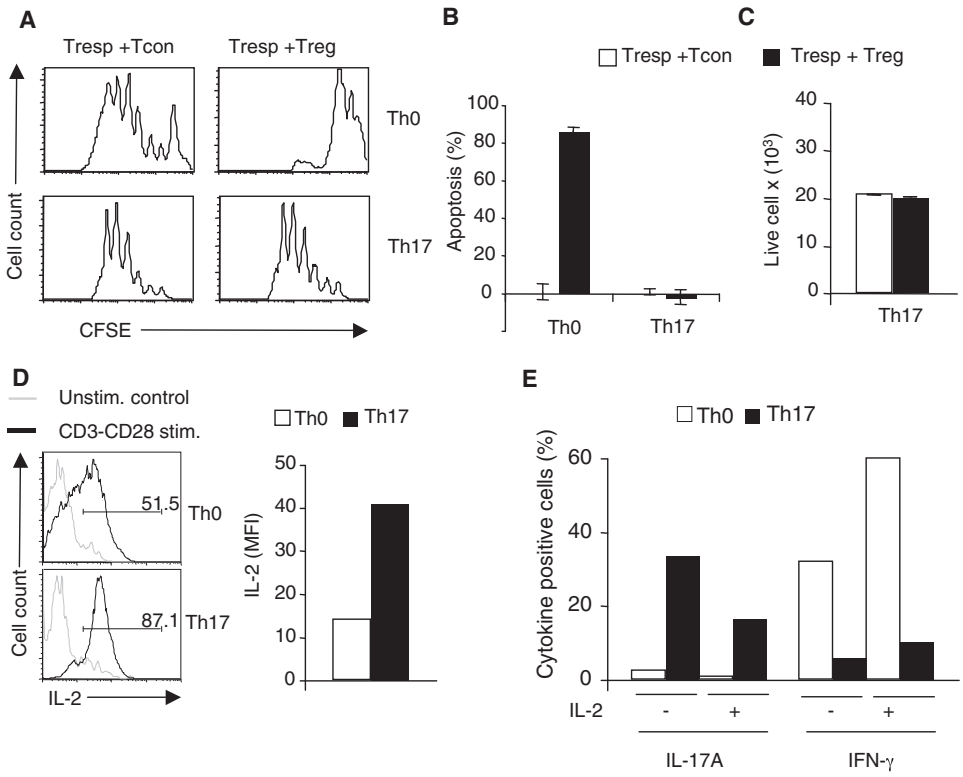

Figure 3. Treg Cells Do Not Induce Apoptosis of Tresp Cells in Th17 Cell Cocultures

(A) Flow cytometric histograms of CFSE dilution of naive CD45.1 CD4 Tresp cells stimulated under nonpolarizing conditions (Th0) (top) or Th17 cell conditions (Th17) (bottom) and cocultured with CD45.2 $CD4^+CD25^-$ (Tcon) cell or $CD4^+CD25^+$ (Treg) cell for 96 hr.

(B) Frequency of apoptotic Tresp cells cultured for 96 hr in Th0 or Th17 cocultures in the presence of Tcon (white bars) or Treg (black bars) cells.

(C) Absolute counts of live Tresp cells in Th17 cocultures in the absence (white bars) or the presence (black) of Treg cells, 3 days after coculture stimulations as in Figure 1B.

(D and E) Flow cytometric histograms of intracellular IL-2 staining (left) and MFI of IL-2 (right) (D) and IL-17A$^+$ or IFN-γ^+ cells (E) cultured for 96 hr in Th0 (white bars) or Th17 (black bars) cell conditions. In (E), 100 U/ml of IL-2 was added as indicated.

loss, and eventually a moribund state. We infected WT B6 mice weighing approximately 20 g with *C. albicans* or PBS sham control (n = 11) as described previously (Conti et al., 2009). We first attempted to test the effect of blocking IL-2 signals, with 0.5 mg α-CD25 (PC61 clone) or isotype control injected intraperitoneally in four mice per group. Two mice were immunosuppressed with cortisone. We observed that even a single injection of α-CD25 rendered mice significantly more susceptible to weight loss on d3 of infection whereas uninfected mice increased in weight (Figure S5A). We assessed the growth of *C. albicans* in tongue preparations and found that the mice receiving PC61 showed significantly increased fungal burden (Figure S5B). As expected, the cortisone-treated control mice exhibited severe disease with rapid weight loss and the highest fungal burden (Figures S5A and S5B). Periodic acid schiff (PAS) staining and histopathological examination of the tongues revealed fungal hyphae in PC61-treated mice but not in isotype control-treated mice even after 5 days of infection (Figure S5C). We also quantified IL-17A in $CD4^+$ cells in cervical lymph nodes (cLN) from infected mice and found that PC61 significantly decreased frequency of IL-17A and IL-17F producers among $CD4^+$ cells compared to isotype control (Figure S5D, first two panels). By contrast, the frequency of IL-2 and IFN-γ producers was low and unaffected by either antibody (Figure S5D, last two panels). These data implied a surprising inhibitory effect of PC61 on the resolution of *C. albicans* infection and IL-17 production during infection. Direct blockade of the IL-2 receptor on Th17 cells would be expected to enhance IL-17A expression and increase fungal clearance. Also, blockade of IL-2 receptor could affect proliferation of T cells or deprive nonlymphoid cells of IL-2. By contrast, PC61 administration is likely to deplete populations of $CD25^+$ cells including natural Treg cells. Removal of a fraction of Treg cells may have resulted in less buffering of IL-2, which would be consistent with the failure of Th17 cell differentiation, more severe disease, and poorer clearance of the fungus during ongoing infection. However, this inference is made cautiously because of many potential effects of blocking IL-2 on a variety of immune cell types. We therefore assessed the frequency of $CD4^+Foxp3^+$ Treg cells and found that it was reduced by almost 50% after the single dose of PC61 antibody before infection but not with the isotype control (Figure S5E). Interestingly, the decrease in the frequency of IL-17A producers correlated well with the decreased frequency of $Foxp3^+$ Treg cells (Figure S5E).

The above results potentially show that depletion of Treg cells could be one factor having an adverse effect on *C. albicans* infection in vivo. We decided to explore this possibility more

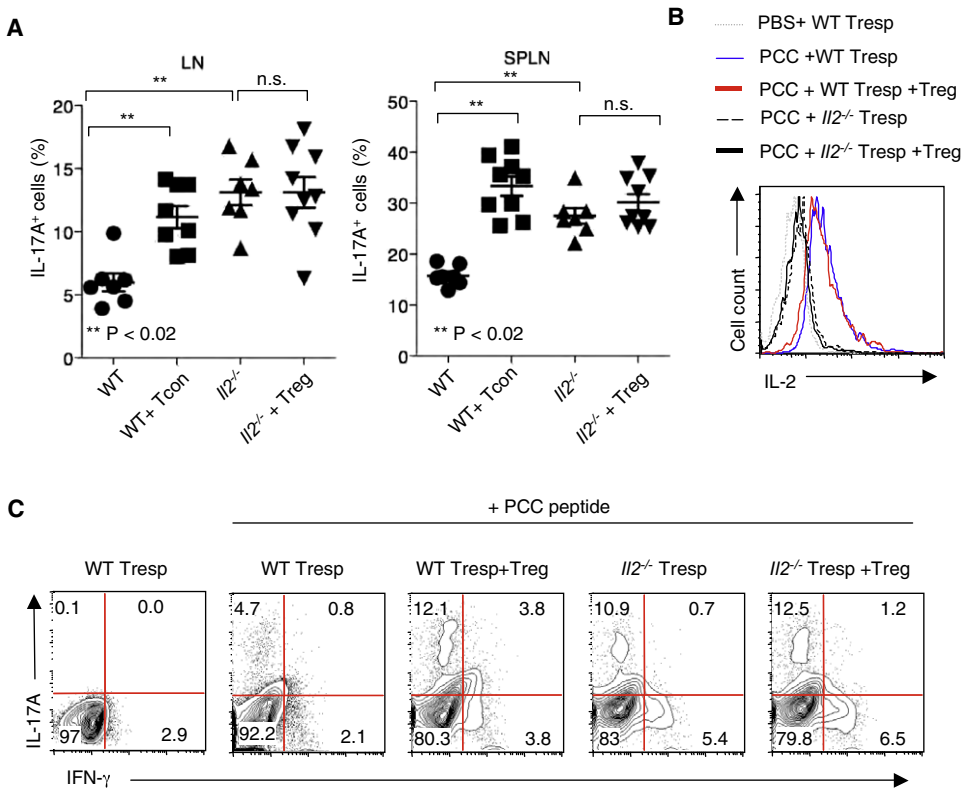

Figure 4. Induction of IL-17 in Tresp Cells Is Dependent on IL-2 Consumption by Treg Cells In Vivo

B10A *Rag2*$^{-/-}$ mice received 6 × 10^5 CFSE-labeled CD4$^+$CD25$^-$ cells from congenic CD45.1 WT or *Il2*$^{-/-}$ 5CC7 *Rag2*$^{-/-}$ TCR tg mice. They also received PBS or 1 × 10^5 fresh Treg cells from donor 5CC7 TCR tg mice. 24 hr later, some mice were immunized with 20 μg of PCC peptide emulsified in 200 μl of CFA. Four days after immunization, PMA-ionomycin-stimulated cells were analyzed for the expression of cytokines.

(A) The frequencies of IL-17A-expressing cells in lymph nodes (LN) or spleen (SPLN) are plotted (gated on CFSE$^+$CD4$^+$ cells). Each data point represents data from each mouse. These data are pooled from three independent experiments showing similar results.

(B and C) Four days after immunization, cells from lymph nodes were analyzed for IL-2 (B) or IFN-γ and IL-17A (C) by flow cytometry (gated on CFSE$^+$CD4$^+$ cells).

carefully. Therefore, we devised a more direct experiment in which *Rag1*$^{-/-}$ immunodeficient mice were reconstituted with naive cells with or without Treg cells followed by acute infection with *C. albicans*. We transferred 1.5 × 10^6 CD45.2 CD4$^+$ CD25$^-$CD44$^-$ naive cells without or with 0.5 × 10^6 CD45.1 CD4$^+$CD25$^+$ Treg cells. Three days after reconstitution, the mice were orally infected with *C. albicans* or oral sham PBS. Cortisone-treated C57BL/6 mice were used as immunosuppressed controls. We assessed the cytokine production 3 days after *Candida* infection in vivo and observed that Treg cells enhanced the differentiation of naive cells into IL-17A-producing cells, whereas they did not alter IL-2 and IFN- γ production in spleen (SPLN) and cLN (Figures 5A and 5B). Correlating with higher percentage of Th17 cells, we found that mice receiving Treg cells recovered from weight loss and showed increased fungal clearance, whereas mice that did not receive them lost weight progressively and had dramatically higher fungal burdens (Figures 5C and 5D). In this adoptive transfer model, we found that naive cells also differentiated in to IFN-γ-producing cells. Although Treg cells did not impact IFN-γ production, the protective or confounding effects of IFN-γ (and Th1 cells) and potential regulatory effects of Treg cells on these cells cannot be ruled out. Therefore, we performed adoptive transfer experiments by using in vitro differentiating Th17 cells (devoid of Th1 cells) in the presence or absence of Treg cells. We reconstituted CD45.2 congenic *Rag1*$^{-/-}$ mice with 4 × 10^5 Tresp cells and 4 × 10^5 Tcon cells or with 4 × 10^5 Tresp cells and 4 × 10^5 Treg cells. These injected cells were derived from Tcon or Treg cell cultures in which CD45.1 Tresp cells were cocultured with CD45.2 Tcon or Treg cells at a ratio of 1:1 under Th17 cell-skewing conditions for 5 days. In this model, we found that only the *Candida*-infected mice exhibited a significant expansion of reconstituted CD45.1$^+$ Tresp Th17 cells in cLN, demonstrating the fungal-specific response in draining lymph nodes (Figure S6A, bottom). Interestingly, we also observed a substantial enrichment of Foxp3$^+$ cells among CD45.1-negative cells in cLN and tongue preparations of the Treg cell recipients compared to Tcon cell recipients, suggesting that the adoptively transferred Treg cells were recruited to the site of infection (Figures S6B and S6C). Consistent with the data from above experiments, we found that mice receiving Treg cells recovered from weight loss, whereas mice that received Tcon cells lost weight progressively (Figure 6A). Importantly, we also found that tongue sections from infected mice, reconstituted with Tresp + Tcon control cells, had dramatically higher fungal burdens as compared to the mice injected with Tresp + Treg cells (Figure 6B). On days 2 and 3 after infection, we isolated

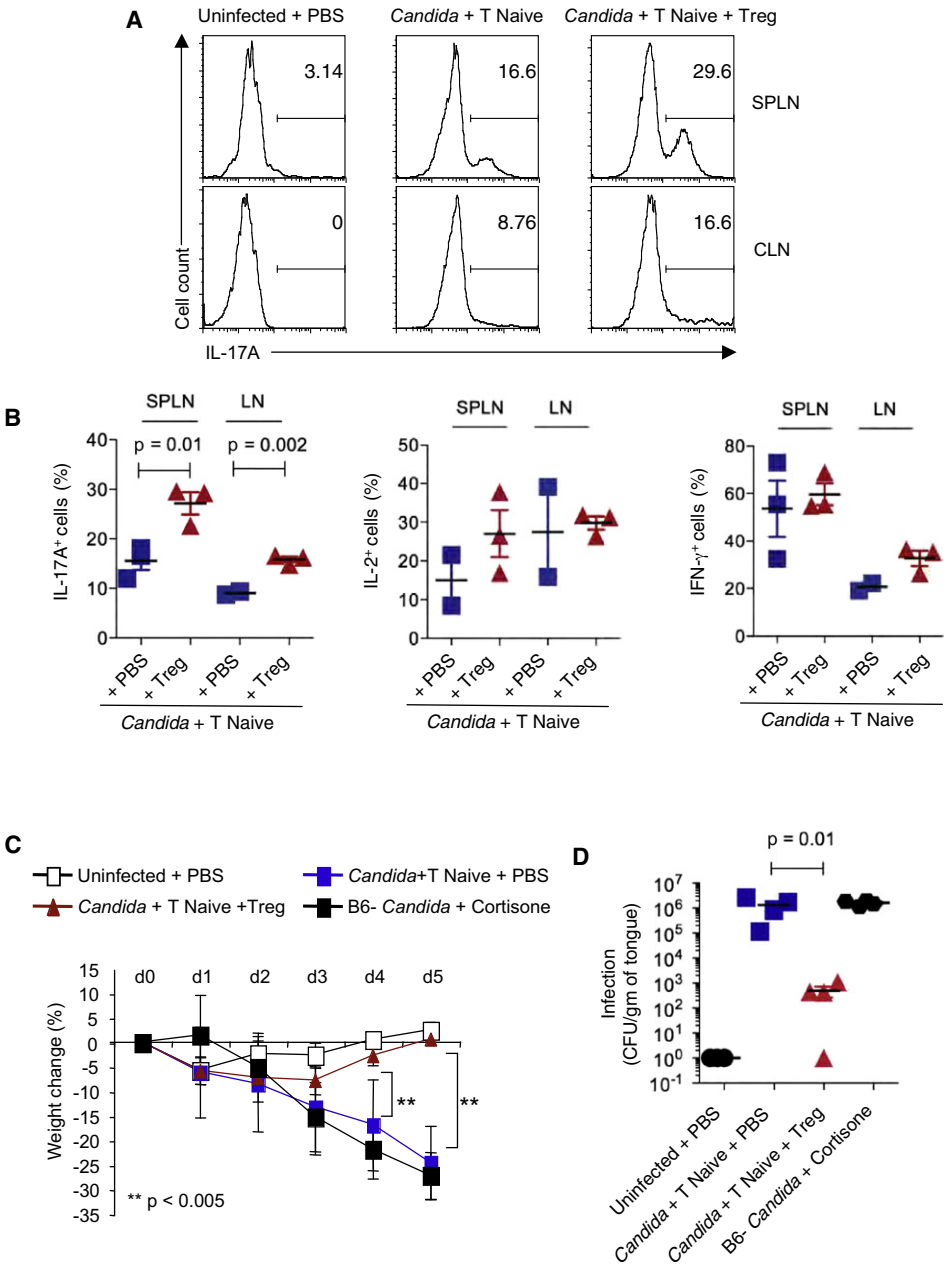

Figure 5. Treg Cells Promote Th17 Differentiation of Naive Cells In Vivo and Protect *C. albicans*-Infected Mice

Rag1$^{-/-}$ mice (n = 14) were reconstituted with CD4$^+$CD25$^-$CD44$^-$ naive cells (T Naive + PBS) without or with CD4$^+$CD25$^+$ Treg cells (T Naive + Treg) and were infected with *Candida*. Some mice were infected with PBS sham controls (Uninfected + PBS). Cortisone-treated C57BL/6 mice serve as immunosuppressed controls.

(A and B) Spleens (SPLN) or cervical lymph nodes (cLN) were harvested on day 3 after infection for IL-17A (A), IL-2, or IFN-γ (B) intracellular staining (gated on CD45.2$^+$CD4$^+$ cells to exclude Treg cells except for uninfected + PBS control).

(C) Percent weight change on indicated days after infection (d0–d5) is shown.

(D) Tongues were harvested on day 5 after infection to assess fungal colony forming units (CFU).

SPLN and cLN to obtain single-cell suspensions, and flow cytometry analyses revealed that CD45.1 Tresp cells in Treg cell recipients contained a clearly increased population of IL-17A-producing T cells compared to Tcon cell recipients (Figure 6C). We also found similar results when we used Tresp cells that were cocultured with Treg cells but were separated from Treg cells before transfer into recipients (Figure S6D). Also, tongue histological immunostaining revealed a higher percentage of RORγt$^+$ cells infiltrating the tongue, indicating an increased Th17 cell response in the Treg cell recipients (Figure 6D, top). We also detected a small fraction of Tbet$^+$ cells (presumably Th1 cells) in the tongue, but their frequency was

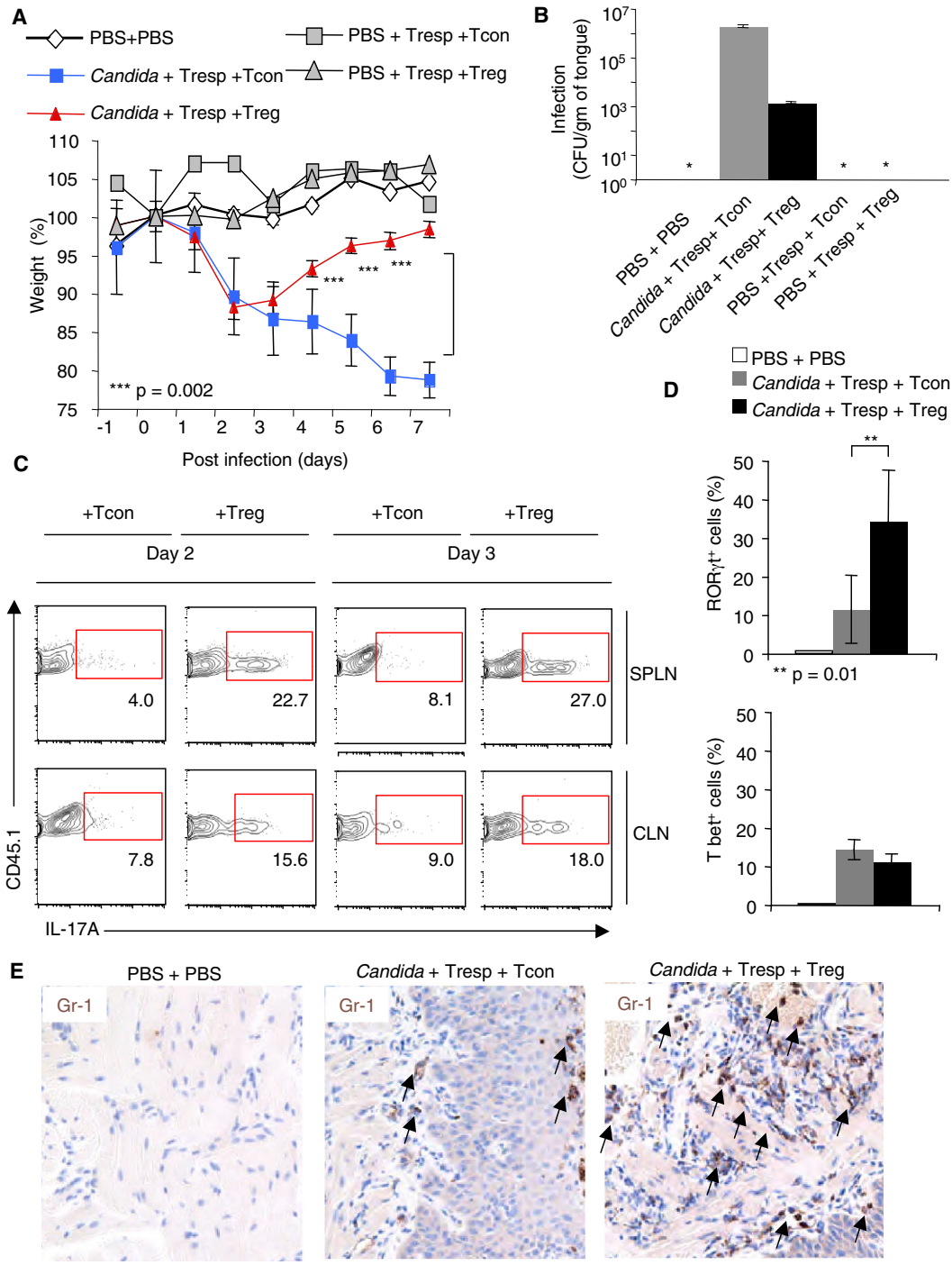

Figure 6. Treg Cells Enhance Th17 Cell Responses and Protect *C. albicans*-Infected Mice

Rag 1$^{-/-}$ CD45.2 mice (n = 15) were reconstituted with Tresp + Tcon or Tresp + Treg cells that were polarized under Th17 cell conditions for 5 days. Tresp cells were obtained from CD45.1 congenic mice and Tcon and Treg cells were obtained from CD45.2 mice. Recipient mice in each group were infected with sham controls or with *C. albicans*.

(A) The percent weight change in mice reconstituted with indicated cells and infected with *C. albicans* on d0.

(B) Mice in indicated groups were sacrificed on day 7 after infection and tongues were harvested. CFU/gm of tongue tissue plated in 10-fold serial dilutions and assessed in triplicates. Mean values ± SEM are plotted. These data are from three independent experiments showing similar results. Asterisks denote zero values for indicated groups.

(C) Mice were reconstituted and infected as in (A). On indicated days postinfection, cells from SPLN and cLN were restimulated with PMA-ionomycin to assess intracellular IL-17A (plots gated on CD45.1 Tresp cells).

(D) The percentage of RORγt (top) and T-bet (bottom)-positive cells in the tongue, as shown by histological immunostaining.

unaffected by the presence of Treg cells (Figure 6D, bottom). One of the main functions of IL-17A in host defense is to recruit neutrophils to the site of infection. Therefore, we examined the neutrophil recruitment in the infected tongue by staining for a neutrophil marker, Gr-1. We found that in the presence of Treg cells, correlating with the increased frequency of Th17 cells, there was a higher frequency of Gr-1-positive neutrophils in the tongue compared to the controls (Figure 6E). These results clearly show that Treg cells do not suppress but decisively enhance a protective Th17 cell response that enabled mice to clear the fungus more efficiently and recover from the infection. To further show that IL-17A produced by Th17 cells is required for the recovery of mice from infection, we also injected several mice with Tresp cells that were cultured with exogenous IL-2 under Th17 cell-inducing conditions. As we demonstrated above, such cells were poor IL-17A producers in vivo (data not shown), and correspondingly, we found that two out of four mice that received IL-2-treated Tresp cells succumbed to the fungus with severe weight loss and death on d3 (data not shown). These data suggest that the capacity of the transferred T cells to produce IL-17A correlates with the protection from the fungus. PAS staining of the tongue revealed extensive fungal growth in control mice that received Tresp cells only whereas mice that received Tresp+Treg cells showed almost little or no evidence of the fungus on day 5 and day 7 after infection (Figure 7). Also, mice reconstituted with Tresp cells treated with IL-2 exhibited widespread fungal growth, infiltration of cells, and extensive tissue damage (Figure 7). Taken together, these results demonstrate that Treg cells could enhance immunoprotective Th17 cell responses and ameliorate infection by *C. albicans*.

The above data raise the issue of whether during Th17 cell-mediated inflammation, Treg cells remain bona fide regulatory cells that can suppress autoimmunity. We therefore carried out control experiments to test the regulatory function of Treg cells in inflammatory bowel disease (IBD) by adoptively transferring Th17 cells similar to those used in *C. albicans* infection model. The cells were mixtures of Thy1.1$^+$ Tresp and Thy1.2$^+$ Tcon or Thy1.2$^+$ Treg cells, polarized under Th17 cell polarizing conditions at a ratio of 1:1 into ten congenic Thy1.2, C.B-17 SCID mice. We found that the mice in Tcon and Treg cell groups started losing weight for the first 2 weeks, showing that Treg cells poorly suppressed the onset of IBD induced by differentiated Th17 cells (Figure S7A). However, around 3 weeks, mice that received Tresp+Treg cells started regaining weight progressively and nearly matched the levels of PBS control mice, whereas the Th17 Tresp+Tcon cells continued to deteriorate (Figure S7A). Interestingly, in the Th0 and Th1 cell model, in which naive cells are used for inducing IBD, Treg cells completely suppressed disease from the onset (Figure S7B). Day 42 colonic sections revealed that mice that received Tresp with Tcon cells had elongated crypts, massive cell infiltration, and a thickened colon wall indicating autoimmune colitis, whereas Treg cell recipients showed no sign of inflammation (Figure S7C). These results demonstrated that the suppressive properties in Treg cells were generally intact and perhaps only slightly attenuated early in acute Th17 cell inflammation in vivo. After cotransfer with Th17 Tresp cells, Treg cells manifested their suppressive functions over time and were capable of efficiently suppressing differentiated Th17 cells and Th17 cell-IBD in vivo. These results demonstrated that the loss of suppressive properties in Treg cells is transient only during acute Th17 cell inflammation in vivo. Treg cells resumed their suppressive functions over time and were capable of efficiently suppressing differentiated Th17 cells and Th17 cell-IBD in a delayed manner in vivo.

DISCUSSION

Although Treg cells have been previously shown to promote IL-17A induction in CD4$^+$ cells, the overall interactions between Treg cells and Th17 cells in vitro and in vivo have remained unclear (Veldhoen et al., 2006; Xu et al., 2007). In particular, our observations on the differentiation of Th17 cells in vitro indicated that even in the presence of saturating amounts of TGF-β, Treg cells still promoted IL-17A upregulation at early and late time points, respectively. Our data show that this most probably occurs by the high capacity of Treg cells to consume IL-2 and decrease overall IL-2 amounts in milieu of Th17 cells. This extends the observation that IL-2 consumption by Treg cells is an important component of their immunological function (de la Rosa et al., 2004; Pandiyan and Lenardo, 2008; Pandiyan et al., 2007). Our current data also indicate that IL-2 consumption by Treg cells is not necessarily suppressive but actually serves a potentially immunoprotective role in promoting Th17 cell differentiation. Previous work has shown that IL-2 inhibits IL-17A production in Th17 cells (Laurence et al., 2007), and we have verified this observation and showed that this effect, which probably influences Th17 cell differentiation, can be controlled by Treg cells. Our current finding mainly reveals that IL-2 restrains IL-17A during an early induction period, and IL-2 consumption by Treg cells may play a less prominent role at later time points or in the maintenance phase of Th17 cell homeostasis. Interestingly, Treg cell-mediated IL-2 consumption did not cause apoptosis or retard proliferation of Th17 cells, which could be due to increased florid production of IL-2 by Th17 cells, compared to Th0 cells. This is consistent with recent quantitative models implying that apoptosis caused by Treg cell-mediated IL-2 consumption may be operational only when IL-2 amounts are limited to close proximity of the responding T cells (Busse et al., 2010; Pandiyan et al., 2007). Our findings contrast with the study of Veldhoen et al. (2006), because we found that Treg cells did not inhibit IL-2 production from Th17 cells. In their study, the sole source of TGF-β was Treg cells, which might have induced IL-17A as well as reduced IL-2 (Gunnlaugsdottir et al., 2005). In our system, Tresp cells were exposed to saturating amounts of exogenous TGF-β and α-CD28 in both the presence and absence of Treg cells and this strong costimulation may have prevented any Treg cell-mediated inhibition of IL-2 synthesis in Tresp cells.

Consistent with the observations that Treg cells promote rather than suppress Th17 cell differentiation, we found that

(E) Mice were reconstituted and infected as in (A). Histological immunostaining for Gr-1 neutrophil marker in tongue on day 3 after infection (brown, denoted by arrows). Microscopic images of the slides viewed at 10× magnification. These results represent data from three independent experiments.

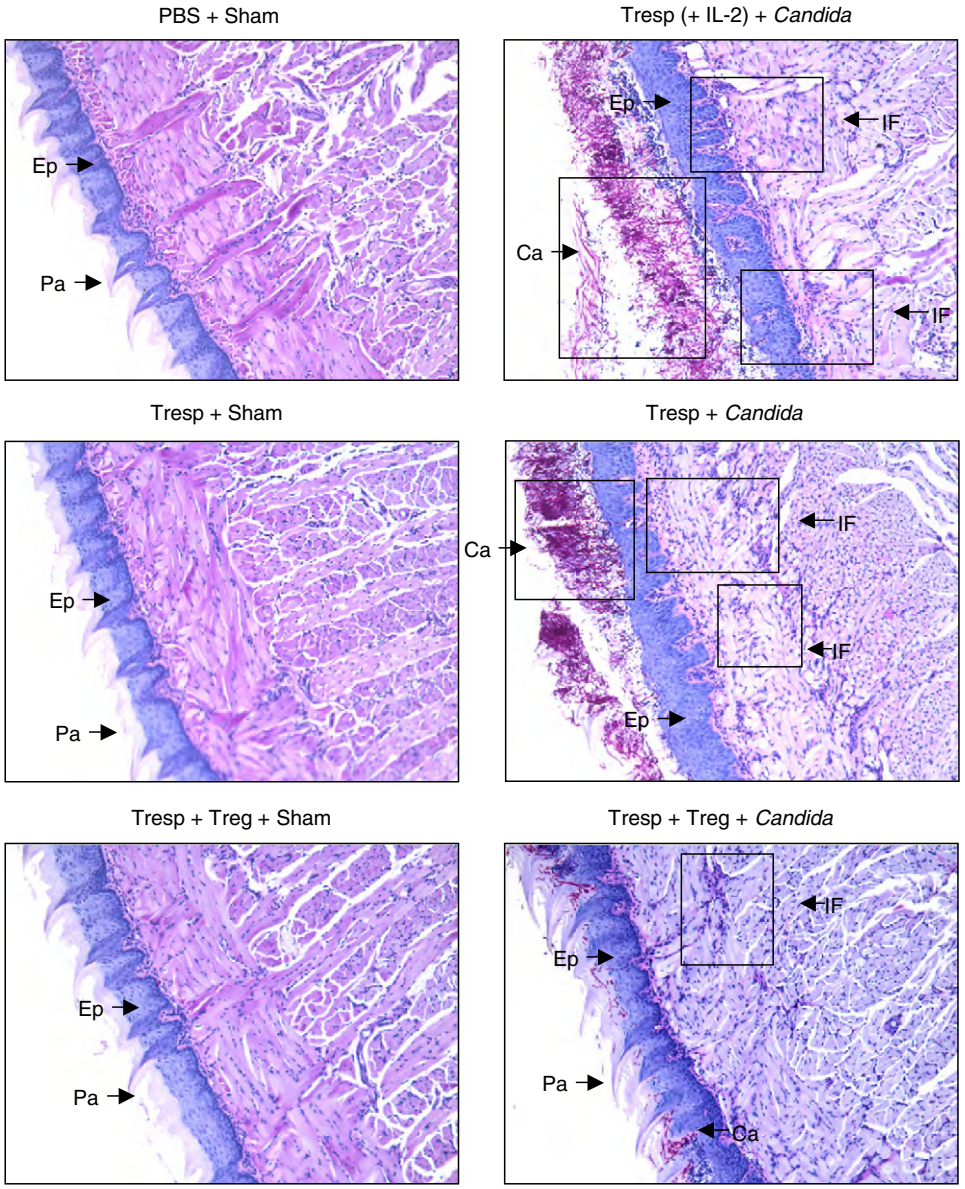

Figure 7. Treg Cells Enhance the Clearance of *C. albicans* in Mice

Histological evaluation of *C. albicans*-infected mice. Mice were reconstituted with indicated cells and infected as in Figure 6. On day 5 after infection, tongues were harvested from mice. Sections of the tongues were stained with PAS to assess inflammation and infiltration (IF) of cells and to detect *C. albicans* (Ca), stained pink in color. Pa and Ep denote papillae and the epithelial layer of the tongue, respectively. Microscopic images of the slides viewed at 10× magnification. Results are representative of two independent experiments.

Treg cells potently enhanced fungal clearance and recovery from oral *C. albicans* infection. Thus, it is now clear that Treg cells play an important role in fighting fungal infections in addition to any effect on maintaining immunological self-tolerance or immune homeostasis. Other recent studies have shown that Treg cells confer protection against viral infections; however, these mechanisms are unrelated to Th17 cell responses (Lanteri et al., 2009; Lund et al., 2008). Our findings may explain the expansion of Treg cells in response to *C. albicans*, resulting in reduced pathology during disseminated candidiasis infection in B7-2-deficient mice (Montagnoli et al., 2002) and why patients with Treg cell defects are prone to *C. albicans* infections (Coutinho and Carneiro-Sampaio, 2008; Roifman, 2000). We believe that the protective effect of Treg cells is largely mediated by an increase in IL-17A production during *C. albicans* infection, because IFN-γ and T-bet expression was unaffected by Treg cells. Nevertheless, IFN-γ is not essential for fungal resistance in this model (Farah et al., 2006). Taken together, these studies strongly support the notion that Treg cells may not exclusively function to "regulate" by suppressing immune responsiveness, but rather cooperate with other helper T cell subsets in immunoprotective functions against infection.

It could be speculated that Treg cell-mediated suppression of inflammation contributed to the recovery of the mice from the disease. However, increased amounts of inflammatory Th17 cell cytokines and enhanced fungal clearance in the presence of Treg cells at least during early infection argue against this possibility. It is notable that we show that Treg cells maintained suppressive capacity because we found that the overall cellularity of Tresp cells was 50% lower in Treg cell recipients than controls on d7 and not on d3 after infection. Also, Treg cells suppressed at later phases in our Th17 cell-IBD model in vivo. Whether Treg cells suppress Th17 cell tissue pathology in a delayed manner is unclear, and likewise the mechanism of this possible delayed suppression is unknown. We are currently investigating these delayed events, which may be Stat-3 dependent as indicated by a recent study (Chaudhry et al., 2009). Previous studies by us and others have implied that Treg cells forfeit suppressive capacity when overwhelming amounts of survival cytokines are available, such as during a severe, ongoing infection that involves toll-like receptor (TLR) signals (Pandiyan et al., 2007; Pasare and Medzhitov, 2003). Our current study demonstrates that Treg cells may have an important antimicrobial role in immunity especially in conjunction with Th17 cell responses. These findings open new avenues in understanding the function of this class of $CD4^+$ T lymphocytes.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 WT or *Rag1*$^{-/-}$ (CD45.1 or CD45.2) and BALB/c (Thy1.1 and Thy1.2) mice were purchased from Jackson Laboratories. C57BL/6, B10.A, CB-17 SCID mice, CD45.1 B10.A mice, WT, and *Il2*$^{-/-}$ 5CC7 TCR transgenic mice in *Rag2*$^{-/-}$ background and *Foxp3*^{gfp} reporter mice were purchased from Taconic Farms (Germantown, NY). CB-17 SCID mice were also purchased from Charles River Laboratories or from Taconic Farms. All mice were maintained at the NIAID animal facility and cared for in accordance with institutional guidelines.

Reagents and Antibodies

Purified α-CD3 (145-2C11), purified α-CD28, α-CD25 (3C7), α-CD4, α-CD25, α-IL-2, α-IL-4, and α-IFN-γ were all purchased from BD Biosciences (San Diego, CA). α-CD25 (PC61), α-IL-17F, α-IL-17-A, α-TNF-α, α-Foxp3, α-CD45.1, α-CD45.2, α-Thy1.1, and α-Thy1.2 antibodies were purchased from eBiosciences (San Diego CA). TGF-β, PE-conjugated IL-22, and IL-6 receptor antibodies were purchased from R&D Systems (Minneapolis, MN). Mouse $CD4^+$ T cell isolation kit II, Anti FITC Multisort kit, and α-Biotin microbeads were purchased from Miltenyi Biotec (Auburn, CA). IL-2 and IL-17A and IL-6 Quantikine ELISA kits, recombinant mouse IL-6, IL-2, and TGF-β were purchased from R&D systems. Mouse cells were cultured in complete RPMI-1640 (Bio-Whittaker) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol.

Cell Purification

Splenocytes were harvested from 5- to 12-week-old mice. $CD4^-$ cells were purified by negative selection with $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Auburn). MACS-sorted $CD4^+$ cells were flow cytometry sorted for naive cells, i.e., $CD4^+CD25^-CD44^{lo}$ cells or Treg cells (>99% purity). In some experiments, we used flow cytometry-sorted $CD4^+CD25^+GFP^+$ Treg cells or $CD4^+CD25^-GFP^-$ Tcon cells from *Foxp3*^{gfp} reporter mice. The purity of $CD44^{lo}CD62L^{hi}CD25^-$ naive cells was more than 99%.

Th17 Cell Differentiation and Coculture with Treg Cells

$CD4^+CD44^{lo}CD62L^{hi}CD25^-$ naive responder T (Tresp) cells (3×10^4) were cocultured in U-bottom 96-well plates with 3×10^4 Tcon cells or 4×10^4 Treg cells in the presence of soluble 1 μg/ml α-CD3 and 2 μg/ml α-CD28 under Th0 or Th17 cell-polarizing conditions for 3–8 days. Tresp cells were derived from congenic CD45.1 or Thy1.1 mice, and Tcon or Treg cells were derived from CD45.2 B6 or Thy1.2 BALB/c mice and so that Tresp cells could be tracked with CD45.1 or Thy1.1 staining (Figure S1). Th0 cells were stimulated only with α-CD3 and α-CD28 with no added cytokines and Th17 cells were polarized with IL-6 (20 ng/ml), TGF-β (2 ng/ml), α-IFN-γ (6 μg/ml), and α-IL-4 (6 μg/ml). The cells showed detectable IL-17A expression around d3 and started to die in the cultures around d8. Therefore, we chose d3 or d4 as early time points and d6 or d7 as late time points to assess cytokine production. Where indicated, Tresp cells were CFSE labeled to assess their proliferation. Cell death analyses were performed based on forward scatter or forward scatter and propidium iodide staining. When indicated, IL-2 (100 U/ml) and α-TGF-β (50 μg/ml) were added at the beginning of stimulation of cocultures. WT or *Il2*$^{-/-}$ cells from B10.A 5CC7 TCR transgenic *Rag2*$^{-/-}$ mice were used as Tresp cells and Tcon or Treg cells were isolated from CD45.1 B10.A mice for this experiment.

IBD Induction by Naive or Th17 Cell Transfer In Vivo

For conventional IBD induction (Th0 IBD), Thy1.2, C.B-17 SCID mice received 4×10^5 fresh CD45.2 $CD25^-CD44^{lo}CD62L^{hi}CD4^+$ congenic cells along with 4×10^5 Thy1.2 Tcon or Treg cells by intraperitoneal (i.p.) injection. For Th17 cell IBD, Thy1.2, C.B-17 SCID mice received Th17 cells that were stimulated and differentiated for 5 days. For this, naive Thy1.1 Tresp cells were cocultured with fresh Tcon or Treg cells at a 1:1 ratio under Th17 cell polarizing conditions and these cocultures were used as the source of Th17 cells. For some experiments, CD45.1 *Rag1*$^{-/-}$ mice were used as recipients and CD45.2 Tresp cells were used as donor cells. Tcon and Treg cells were derived from congenic CD45.1 mice in these experiments. The weight of the recipient mice was monitored in a blinded fashion. SPLN, MLN, and the gut for isolation of lamina propria mononuclear cells (LPMC) were harvested at indicated time points after induction for phorbol myristate acetate (PMA)-ionomycin restimulation and intracellular cytokine analyses.

C. albicans Infection in Mice

Experiments with an oral *C. albicans* mouse model were performed at the University of Buffalo. All protocols were approved by SUNY Buffalo Institutional Animal Care and Use Committee. Age- and sex-matched C57BL/6 mice were infected and individually caged after infection, as previously described (Conti et al., 2009; Kamai et al., 2001). In brief, they were anesthetized with a mixture of ketamine (100 mg/ml):xylazine (20 mg/ml) (2:1) solution diluted 5-fold with sterile saline. "Y" μl of anesthetic mixture was administered, Y being calculated according to weight of mouse (Y = weight of the mouse [gm] × 3.9 + 70). They were infected under anesthesia by placing a 0.0025 g cotton ball saturated with 2×10^7 *C. albicans* (CAF2-1) blastospores sublingually for 90 min. They were treated with 225 mg/kg cortisone acetate (Sigma-Aldrich) if indicated. For experiments involving immunodeficient mice, CD45.2 *Rag1*$^{-/-}$ mice were reconstituted with T cells 3–5 days before infection. We also performed one experiment with C.B-17 SCID mice recipients and Thy1.1 Tresp donor cells and Tcon and Treg cells from Thy1.2 mice.

Histology and Intracellular Staining of Cytokines

For immunocytochemical hematoxylin and eosin (H&E) staining, tissues were washed with PBS, fixed with 10% formalin overnight, and suspended in 70% ethanol to prevent overfixation. Paraffin sectioning and immunostaining of paraffin sections were performed by Histoserv, Inc. (MD). For single-cell staining, cells were cultured as above, washed in PBS, and fixed with CytoFix-Cytoperm kit (BD BioSciences). Before fixation, cocultures were restimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4–6 hr, with brefeldin-A (10 μg/ml) added in last 2 hr.

Flow Cytometry

Data was acquired with BD FACS Calibur cytometers and were analyzed with FlowJo 8.8.4 software.

Statistical Analyses

p values were calculated by Student's t test in Microsoft Excel software with unpaired, two-tailed distribution and two-sample equal variance parameters or Mann-Whitney test in Prism 4.0 (GraphPad Software, Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.immuni.2011.03.002.

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